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2-Aminoethoxydiphenyl borate (2-APB) antagonises inositol 1,4,5-trisphosphate-induced calcium release, inhibits calcium pumps and has a use-dependent and slowly reversible action on store-operated calcium entry channels

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Abstract

The action of 2-aminoethoxydiphenyl borate (2-APB) on Ca^{2+} signalling in HeLa cells and cardiac myocytes was investigated. Consistent with other studies, we found that superfusion of cells with 2-APB rapidly inhibited inositol 1,4,5-trisphosphate ($InsP_3$)-mediated Ca^{2+} release and store-operated Ca^{2+} entry (SOC). In addition to abrogating hormone-evoked Ca^{2+} responses, 2-APB could antagonise Ca^{2+} signals evoked by a membrane permeant $InsP_3$ ester. 2-APB also slowed the recovery of intracellular Ca^{2+} signals consistent with an effect on Ca^{2+} ATPases. The inhibitory action of 2-APB on $InsP_3$ receptors ($InsP_3$Rs), SOC channels and Ca^{2+} pumps persisted for several minutes after washout of the compound. Application of 2-APB to unstimulated cells had no effect on subsequent Ca^{2+} responses suggesting that it has a use-dependent action. Mitochondria in cells treated with 2-APB showed a rapid and slowly reversible swelling. 2-APB did not cause the mitochondria to depolarise, but it reduced the extent of mitochondrial calcium uptake. Although 2-APB has been demonstrated not to affect voltage-operated Ca^{2+} channels or ryanodine receptors, we found that it gave a concentration-dependent long-lasting inhibition of Ca^{2+} signalling in electrically-stimulated cardiac myocytes, where $InsP_3$Rs and SOC channels do not play a significant role. Our data suggest that 2-APB has multiple cellular targets, a use-dependent action, is difficult to reverse and may affect Ca^{2+} signalling in cell types where $InsP_3$ and SOC are not active.

Keywords: 2-Aminoethoxydiphenyl borate; Ca^{2+} signalling; $InsP_3$

1. Introduction

Ca^{2+} release from intracellular stores is mediated by a variety of intracellular messengers and channels [1]. One widely-expressed family of intracellular Ca^{2+} channels is activated by the lipid-derived messenger, inositol 1,4,5-trisphosphate ($InsP_3$). Binding of $InsP_3$ to its receptors ($InsP_3$Rs) mobilises Ca^{2+} from the lumen of organelles such as the endoplasmic/sarcoplasmic reticulum (E/SR) and Golgi [2,3]. At present, there is a paucity of available antagonists that can be used to probe the contribution of $InsP_3$Rs in cellular responses. One of the most commonly used $InsP_3$R antagonists is heparin. This compound is limited because it has multiple actions including uncoupling G-protein signalling and activating ryanodine receptors (RyRs) (for reviews see [4,5]). Furthermore, heparin is not membrane permeant, and therefore has to be injected or infused into cells from micropipettes, although it has been suggested that low molecular weight heparin species may cross the plasma membrane and thus inhibit $InsP_3$Rs within intact cells [6]. Xestospongins, which were first described as relatively specific membrane-permeant $InsP_3$R antagonists by Pessah and colleagues [7], have been employed many times to prove whether $InsP_3$Rs are involved in particular responses. Although there are many examples of xestospongin inhibiting Ca^{2+} signalling, its mechanism of action

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has not been fully elucidated. Furthermore, xestospongin is expensive, often slow to act and has not been universally successful (e.g. [8]).

2-Aminoethoxydiphenyl borate (2-APB) was introduced as an $InsP_3R$ antagonist by Maruyama et al. [9]. It has subsequently been used in many studies to probe the contribution of $InsP_3Rs$ in the generation of cellular Ca^{2+} signals (for review see Ref. [10]). The initial study of 2-APB showed that it evoked a concentration-dependent inhibition of $InsP_3$-induced Ca^{2+} release from mouse cerebellar membranes. The mechanism by which 2-APB inhibits $InsP_3R$ activity is unclear, although it does not diminish $InsP_3$ binding [9,11–13].

Unlike xestospongins, which can inhibit both $InsP_3Rs$ and RyRs, 2-APB may not have any action on the latter type of channel. It did not affect caffeine-activated Ca^{2+} release from striated muscle vesicles [9] or toad sinus venosus [14]. Furthermore, in smooth muscle, 2-APB inhibited contractile responses to $InsP_3$-generating stimuli, but not those triggered by KCl-induced depolarisation [9,15,16] suggesting that there was no effect on voltage-operated Ca^{2+} entry or the contractile machinery. Even though 2-APB may have no effect on RyRs or voltage-operated Ca^{2+} channels, it is clearly not entirely specific for $InsP_3Rs$ [10] and in some cell types inhibition of $InsP_3$-induced Ca^{2+} release is not observed at all (e.g. [17]). Probably the most consistent action of 2-APB is to block store-operated Ca^{2+} entry (SOC) [10]. The inhibition of SOC is not due to action of 2-APB on $InsP_3Rs$ [18], since it occurs in cells not expressing those channels [19–23], or in processes that do not require functional $InsP_3Rs$ such as *Drosophila* phototransduction [24]. Furthermore, 2-APB rapidly blocks SOC by acting at extracellular sites [19,20,25]. In addition, it has been shown to inhibit sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCAs) [11,26], thus promoting the gradual release of Ca^{2+} stores.

Despite the lack of specificity of 2-APB, it remains a widely-used inhibitor of intracellular Ca^{2+} signalling, and it is therefore important to elucidate its intracellular targets and mechanism of action. In the present study, we examined the ability of 2-APB to affect Ca^{2+} signalling in HeLa cells and cardiac myocytes. Although it clearly has a rapid antagonistic action on $InsP_3$-evoked Ca^{2+} release, it appears to have a use-dependent action on multiple targets and is poorly reversible.

2. Materials and methods

2.1. HeLa cell culture

HeLa cell culture was performed as described previously [27]. All experimental procedures were carried out at room temperature (20–22 °C). Prior to imaging, the culture medium was replaced with an extracellular medium (EM) containing (mM): NaCl, 121; KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.8; $NaHCO_3$, 6; D-glucose, 5.5; HEPES, 25; pH 7.3. Histamine, ATP, thapsigargin and ionomycin were obtained from Sigma. All fluorescent dyes were obtained from Molecular Probes (OR, USA). Statistics were performed using Student's *t*-test (GraphPad Prism, San Diego, CA, USA). The $InsP_3$ ester was made by Dr. Stuart Conway and Professor Andrew Holmes (Department of Chemistry, University of Cambridge, UK). Please contact the authors for details of the synthesis.

2.2. Video imaging

Measurement of cytosolic calcium in HeLa cells was performed by monitoring Fura2 fluorescence of cells adhered to glass coverslips using either a Spex or a Perkin-Elmer imaging system. Fura2 was loaded into the cells by incubation with 2 μM Fura2 acetoxymethyl ester (30 min incubation followed by a 30-min period for de-esterification). For the Spex system, cells on coverslips were mounted on a Nikon Diaphot inverted epi-fluorescence microscope. Fluorescent images were obtained by alternate 40 ms excitation at 340 and 380 nm using twin xenon arc lamps each coupled to a spex monochrometer (Spex Industries Edison, NJ, USA), with the wavelengths being switched by a rotating chopper mirror (Glen Creston Instruments, Stanmore, UK). Emission signals at 510 nm were collected using an intensified charge-coupled device video camera (Photonics Science, Robertson, UK) and filtered with a 200 ms time constant (Spex system) prior to off-line storage for analysis using an Imagine image processing system (Synoptics, Cambridge, UK) as described previously.

With the Perkin-Elmer system, a single glass coverslip with adherent cells was mounted on the stage of a Nikon Diaphot 300 inverted epi-fluorescence microscope coupled to a xenon arc lamp (Nikon) light source. Fluorescence images were obtained with alternate excitation at 340 and 380 nm, selected for using either a Sutter filter wheel (340HT15 and 380HT15, Sutter Industries), or a Spectramaster II monochrometer. Emitted light was filtered at 510 nm and collected by a cooled Astrocam digital camera. The acquired images were stored and subsequently processed off-line with Ultraview software (Perkin-Elmer Life Sciences Ltd., Cambridge, UK).

2.3. Confocal microscopy of mitochondrial membrane potential and calcium uptake

HeLa cells were loaded with tetramethyl rhodamine ethyl ester (TMRE; 0.1 μM for 20 min) and a median section through the cell was imaged with 60× (1.4 NA) objective on a BioRad MRC1024 laser scanning confocal microscope (LSCM) (excitation 514 nm and emission >525 nm). For mitochondrial calcium uptake experiments, HeLa cells were loaded with Rhod-2 (1 μM for 30 min followed by a further 30 min for de-esterification). A median section through the cells was imaged (images collected every 2.5 s) using a BioRad MRC1024 LSCM (excitation 514 nm and emission >525 nm).

2.4. Cardiomyocyte isolation and calcium measurement

Rat atrial myocytes were isolated from male Wistar rats by an enzymatic isolation method described earlier [28]. Single cells were kept in an EM containing (in mM): NaCl, 135; KCl, 5.4; $MgCl_2$, 2; $CaCl_2$, 1; glucose, 10; HEPES, 10; pH 7.35. All experiments were performed at room temperature (20–22 °C). Cells were loaded with 4 μM Indo1 (30 min with Indo1/AM followed by a 30-min de-esterification period). Whole cell fluorescence in electrically paced (0.3 Hz) cells was imaged using a photometric imaging system (Perkin-Elmer Life Sciences, Cambridge, UK) sampling at 200 Hz. Cells were electrically paced with a pair of field electrodes (1 Hz, 40 V, 2 ms duration).

3. Results

3.1. 2-APB inhibits $InsP_3$-evoked Ca^{2+} release

To determine that 2-APB was able to enter cells and inhibit Ca^{2+} release, Fura2-loaded HeLa cells were stimulated with a membrane-permeant $InsP_3$ ester that directly activates $InsP_3$Rs following de-esterification [29,30]. The $InsP_3$ ester (20 μM) by itself typically evoked a ~10-fold increase of the cytosolic Ca^{2+} concentration (Ca^{2+}_{cyt}). Application of 2-APB (100 μM) prior to $InsP_3$ ester completely inhibited the Ca^{2+}_{cyt} elevation (Fig. 1A). 2-APB gave a similar inhibition of Ca^{2+}_{cyt} responses within cells stimulated in extracellular Ca^{2+}-free medium (Fig. 1B). Addition of 2-APB to cells already responding to $InsP_3$ ester caused the rapid decline of Ca^{2+}_{cyt} back to basal levels (Fig. 1C). Typically, 2-APB inhibited the $InsP_3$ ester-induced Ca^{2+}_{cyt} elevation after a delay of only a few seconds. These data suggest that 2-APB can readily enter HeLa cells and inhibit $InsP_3$Rs.

Similar to its effects on $InsP_3$ ester-induced Ca^{2+} signals, 2-APB inhibited responses evoked by the $InsP_3$-generating agonist histamine. The degree of inhibition was dependent on the stimulating histamine concentration with the responses to 100, 10 and 1 μM histamine being reduced to 28±1, 5±1 and 3±0.1% of a control response, respectively (Fig. 2 and data not shown). With 1 or 10 μM histamine, 2-APB reduced both the amplitude of Ca^{2+}_{cyt} increases and also the proportion of responsive cells (Fig. 2E).

Ca^{2+}_{cyt} signals evoked by another $InsP_3$-generating stimulus, ATP, were also inhibited by 2-APB. However, 2-APB appeared to have a more substantial inhibitory effect on ATP-evoked responses than those observed with histamine, as it completely prevented Ca^{2+}_{cyt} elevation by 1, 10 or 100 μM ATP (Fig. 3A–D and data not shown). ATP is a weaker agonist than histamine on HeLa cells. The concentrations of histamine and ATP required for half-maximal Ca^{2+} signals are ~2.5 and 10 μM, respectively (data not shown). However, 100 μM of either agonist produced similar maximal Ca^{2+}_{cyt} increases (Fig. 3E). It is therefore surprising that 100 μM 2-APB was able to completely inhibit responses to a maximal ATP dose whilst sub-maximal concentrations of histamine (i.e. 1 and 10 μM) could evoke signals (Figs. 2 and 3).

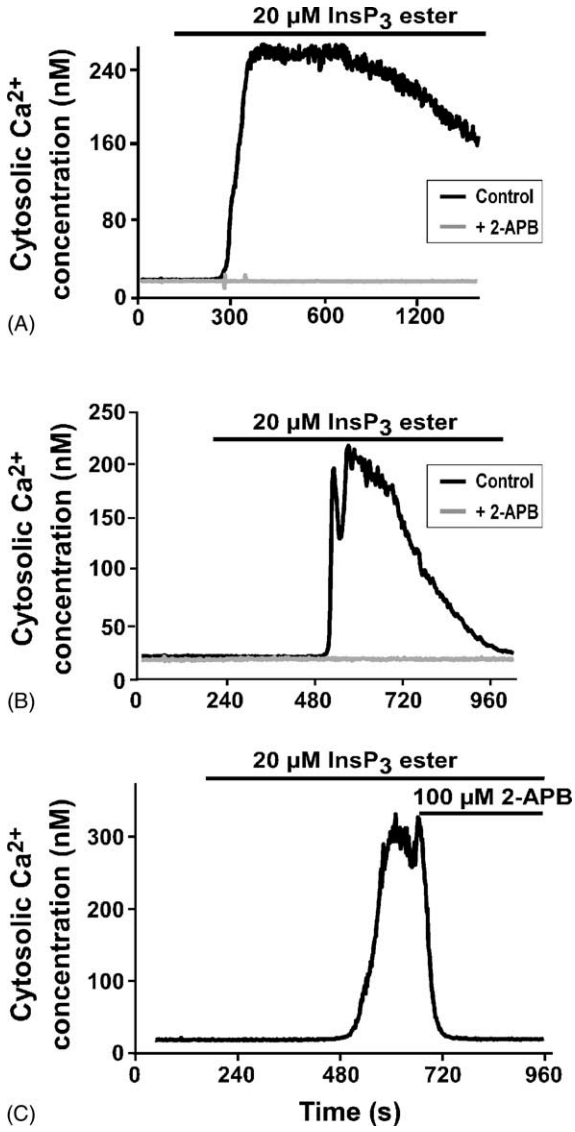

Fig. 1. 2-APB inhibits Ca^{2+} signals evoked by a membrane-permeant $InsP_3$ ester. Panels A–C illustrate the inhibition of Ca^{2+}_{cyt} responses evoked by application of membrane-permeant $InsP_3$ ester (20 μM). The $InsP_3$ ester was applied at the time shown by the horizontal bars. For panels A and B, 100 μM 2-APB was applied 2 min before the $InsP_3$ ester, to allow for equilibration. The experiments in panels A and C were performed with cells in Ca^{2+}-containing extracellular medium. The experiment in panel B was performed with cells in a nominally Ca^{2+}-free medium. The traces shown are from individual cells, and are typical of the responses of at least 20 cells for each condition analysed in three independent experiments.

We did not investigate the effects of 2-APB concentrations higher than 100 μM since they caused a pronounced increase of basal Ca^{2+}_{cyt}, consistent with earlier reports of SERCA inhibition [11,26]. Concentrations of 2-APB lower than 100 μM produced curious effects. With maximal histamine concentrations, 1 and 10 μM 2-APB caused inhibi-

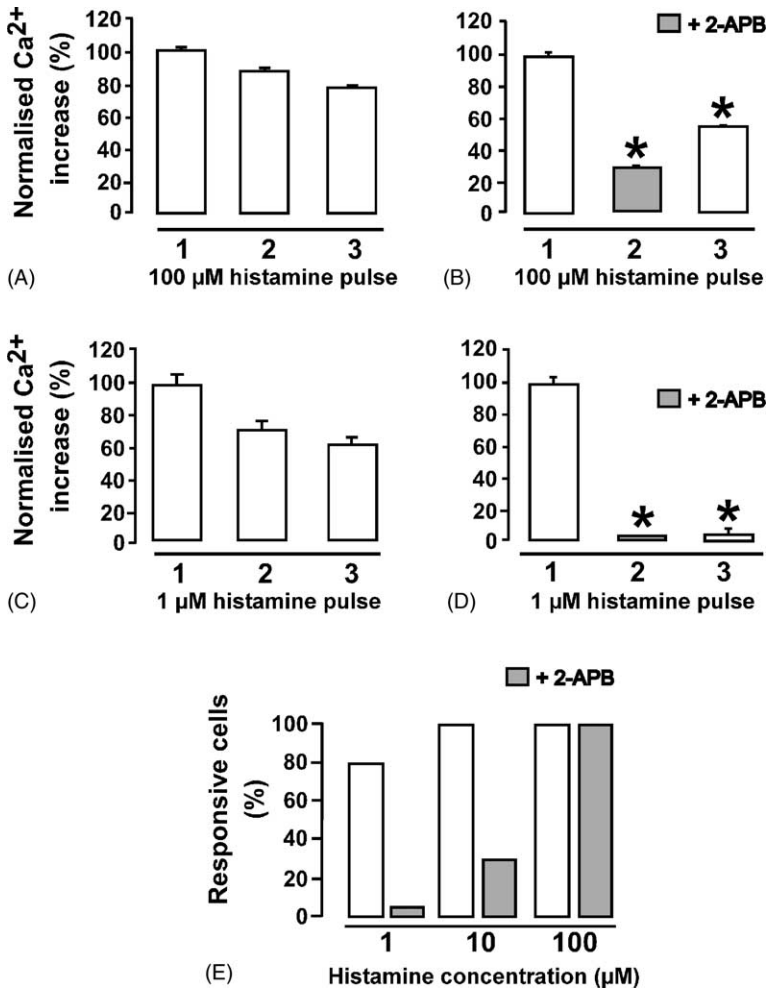

Fig. 2. 2-APB inhibits Ca^{2+} signals evoked by histamine. Panels A and C depict the normalised Ca^{2+}_{cyt} response of HeLa cells to three consecutive pulses of histamine (1 min agonist application followed by a 10 min continuous wash between pulses). Panels B and D illustrate the effect of 100 μM 2-APB on histamine-evoked Ca^{2+} responses (2-APB was added 2 min prior to the second histamine pulse, and maintained during the histamine application). For panels A–D, the Ca^{2+}_{cyt} responses are shown as normalised values, calculated as a percentage of the control response (i.e. pulse 1 = 100%). The data represent the averaged response (mean ± S.E.M.) of at least 30 cells from three independent experiments for each histamine concentration. Panel E indicates the effect of 2-APB on the proportion of cells responsive to the histamine concentrations shown. The asterisk (∗) indicates significantly different from control ($P < 0.05$).

tion of the Ca^{2+}_{cyt} responses. With lower histamine concentrations, 1 and 10 μM 2-APB potentiated Ca^{2+}_{cyt} signals. For example, the Ca^{2+}_{cyt} response evoked by 1 μM histamine was increased from 69 ± 12 nM ($n = 20$) to 120 ± 15 nM ($n = 30$) by concurrent application of 10 μM 2-APB (data not shown).

3.2. 2-APB is poorly reversible and has a 'use-dependent' action

The inhibition of histamine- and ATP-evoked Ca^{2+}_{cyt} signals by 2-APB did not fully reverse on washout of 2-APB. Using pulsatile application of histamine (1 min application followed by a 10 min washout), it appeared that 2-APB (100 μM) not only inhibited responses when it was present, but also decreased the amplitude of Ca^{2+}_{cyt} signals observed with successive agonist additions (Figs. 2 and 3). The long-lasting inhibitory effect of 2-APB only occurred if it was applied at the same time as an $InsP_3$-generating agonist. If 2-APB was given by itself, it did not cause any decrement of subsequent agonist-evoked Ca^{2+} signals beyond the normal desensitisation observed with HeLa cells (Fig. 4). The requirement for concurrent cell stimulation for the prolonged inhibitory effect of 2-APB is reminiscent of the use-dependent action of other agents such as ryanodine [31], and may suggest that it binds more readily to activated channels.

Histamine- and ATP-evoked responses declined in the repetitive agonist application protocol even in the absence of 2-APB (Figs. 2 and 3). This was most likely due to the progressive desensitisation of agonist-mediated Ca^{2+} signalling. However, both the acute and sustained inhibitory effects of 2-APB were obvious and significant despite the on-going desensitisation to agonists.

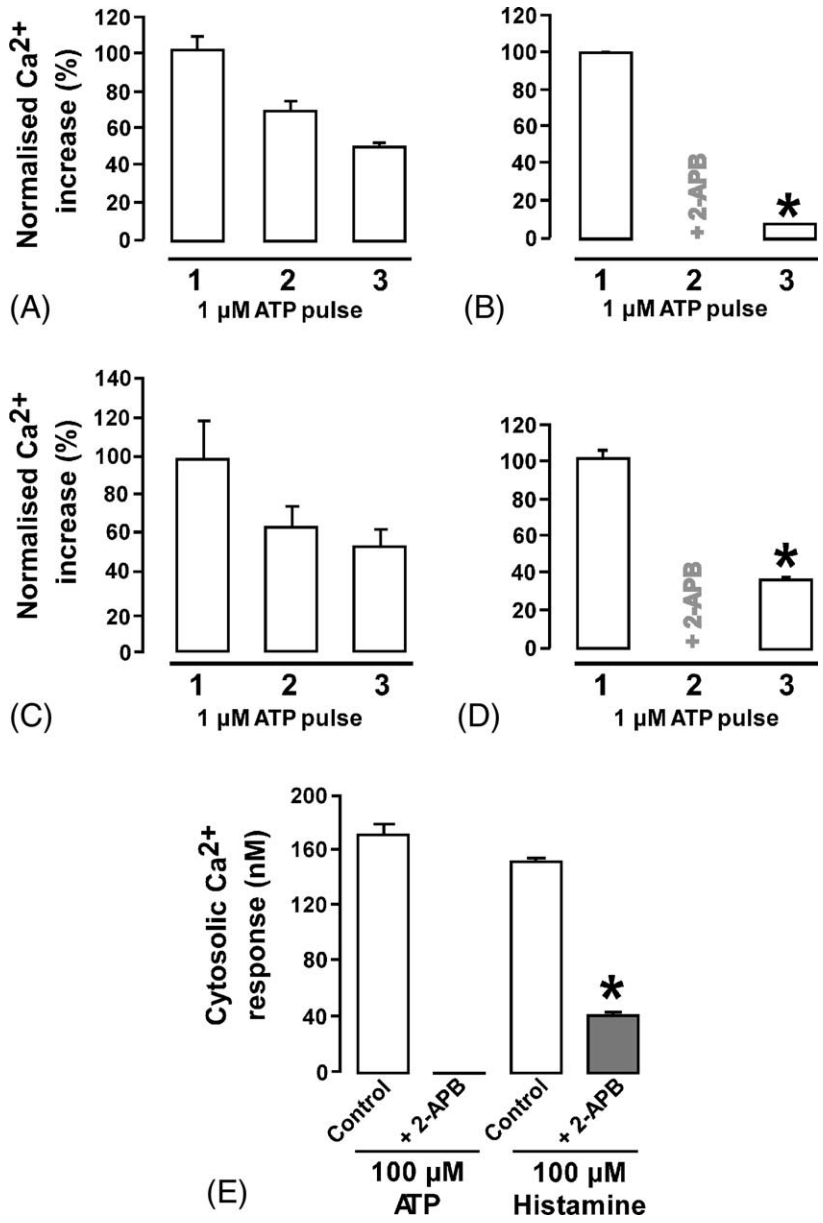

Fig. 3. 2-APB inhibits Ca^{2+} signals evoked by ATP. Panels A and C depict the normalised Ca^{2+}_{cyt} response of HeLa cells to three consecutive pulses of ATP (1 min agonist application followed by a 10 min continuous wash between pulses). Panels B and D illustrate the effect of 100 μM 2-APB on ATP-evoked Ca^{2+} responses (2-APB was added 2 min prior to the second ATP pulse, and maintained during the ATP application). For panels A–D, the Ca^{2+}_{cyt} responses are shown as normalised values, calculated as a percentage of the control response (i.e. pulse 1 = 100%). The data represent the averaged response (mean ± S.E.M.) of at least 30 cells from three independent experiments for each ATP concentration. The asterisk (∗) indicates significantly different from control ($P < 0.05$). Panel E shows that maximal concentrations of ATP and histamine (100 μM of each) evoked similar Ca^{2+} increases, yet 2-APB (100 μM) was a more potent inhibitor of ATP responses.

In addition to its inhibitory effect on $InsP_3$-induced Ca^{2+} release, 2-APB has been shown to be a potent blocker of SOC-mediated Ca^{2+} influx [10]. 100 μM 2-APB immediately and completely curtailed Ca^{2+}_{cyt} increases due to Ca^{2+} influx triggered by discharge of intracellular Ca^{2+} stores with thapsigargin (2 μM; data not shown). Similar to the effect on histamine-induced Ca^{2+} signals, the inhibition of SOC did not reverse fully during a 5-min wash period (Fig. 5A–D). Furthermore, 2-APB also had a use-dependent effect on Ca^{2+} influx. If 2-APB was applied to cells prior to stimulation with thapsigargin, it did not inhibit SOC-mediated Ca^{2+} entry (Fig. 6A and B). However, once SOC was activated following Ca^{2+} store depletion, one brief pulse of 2-APB produced a long-lasting and substantial reduction of Ca^{2+} influx (Fig. 6C and D).

Another significant and long-lasting effect of 2-APB was to decrease the rate of decline of Ca^{2+}_{cyt} signals. Using the protocol shown in Fig. 5A, where SOC-mediated Ca^{2+}

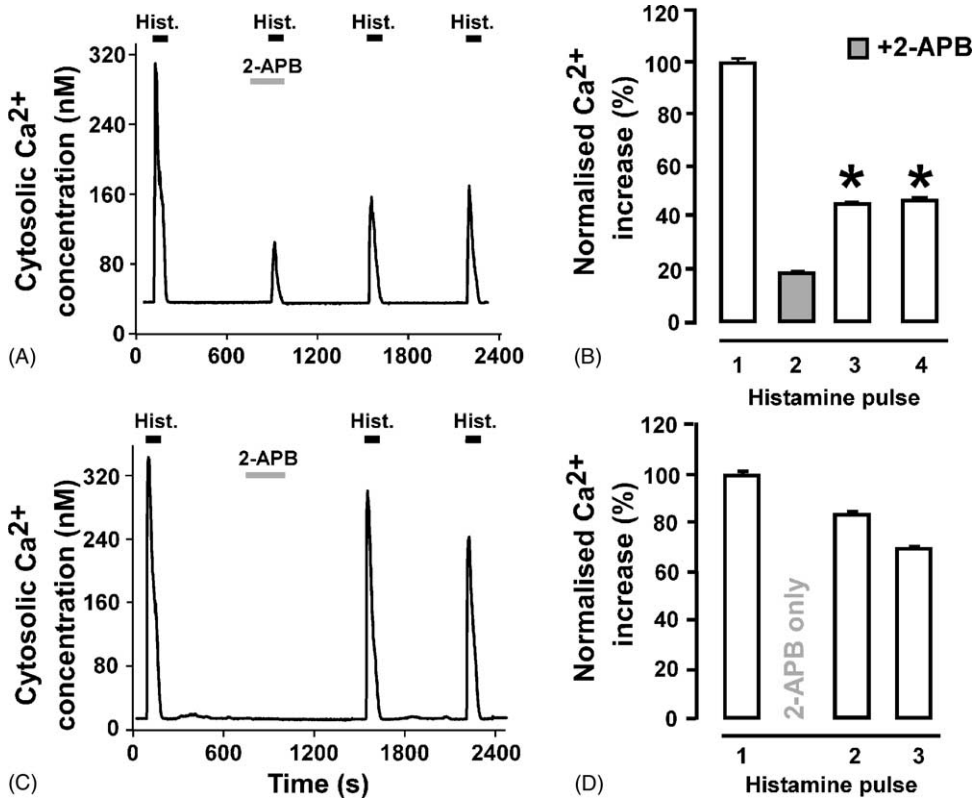

Fig. 4. The inhibition of histamine-evoked Ca^{2+} signals by 2-APB is use-dependent and poorly reversible. Panels A and C depict the response of individual HeLa cells to pulsatile application of histamine. For panel A, histamine was applied four times (1 min application and ~10 min washout; shown by the black bars), and 2-APB was added 1 min before and during the second histamine perfusion. Panel C depicts a similar experiment to that in A, except that 100 μM 2-APB was applied to the cell in the absence of histamine. Panels B and D show the normalised data (mean ± S.E.M.) from at least 30 cells from three independent experiments for each condition. The asterisk (∗) indicates significantly different from control ($P < 0.05$).

influx was triggered by pulsatile application of extracellular Ca^{2+} following discharge of Ca^{2+} stores with thapsigargin, it was observed that the mono-exponential rate constant for the recovery of Ca^{2+}_{cyt} was decreased by 2-APB (Fig. 5E). In addition to reducing the rate constant for decline of Ca^{2+}_{cyt} for the Ca^{2+} pulse when it was applied, 2-APB also reduced the recovery of Ca^{2+}_{cyt} for subsequent Ca^{2+} pulses after it had been washed out (Fig. 5E). Since the SERCA pumps were inhibited by thapsigargin in these experiments, the decline of Ca^{2+}_{cyt} recovery must be due to inhibition of the other Ca^{2+} removal pathways, such as plasma membrane Ca^{2+} ATPases (PMCAs) and mitochondria.

Previous evidence has suggested that 2-APB can interact with mitochondria and prevent them releasing sequestered Ca^{2+}, possibly by an inhibitory action on the Na^{+}/Ca^{2+} exchanger [19]. We observed that 100 μM 2-APB caused mitochondria to reversibly swell and change shape (Fig. 7A). It does not cause them to depolarise (Fig. 7B), but it did reduce the extent of Ca^{2+} uptake into the mitochondrial matrix (Fig. 7C).

The prolonged inhibition of SOC may account for the failure of histamine- and ATP-evoked Ca^{2+}_{cyt} signals to recover following 2-APB washout (Figs. 2 and 3). Reduction in SOC would have prevented the Ca^{2+} stores from fully refilling during the inter-stimulation interval. Consistent with this, it was found that the amount of ionomycin-releasable Ca^{2+} in the intracellular stores was decreased by 2-APB. Following two brief histamine pulses (1 min agonist application followed by a 10 min wash), the peak ionomycin-induced Ca^{2+} signal was found to be ~60% larger than the first histamine application (Fig. 8A). If 100 μM 2-APB was applied during the second histamine pulse the subsequent ionomycin response was decreased by ~40% (Fig. 8B).

As mentioned above, we chose to investigate the action of 100 μM 2-APB since this was the highest concentration that did not cause a significant change of Ca^{2+}_{cyt} by itself. However, even though there was no change in Ca^{2+}_{cyt}, this concentration of 2-APB did enhance loss of Ca^{2+} from the intracellular Ca^{2+} pool. Removal of Ca^{2+} from medium in which HeLa cells are bathed causes the progressive depletion of the intracellular stores, probably due to passive Ca^{2+} leakage and the inability of SOC to replenish the lost Ca^{2+} (data not shown). 2-APB exacerbated the rate of decline of the Ca^{2+} stores when cells were placed in Ca^{2+} free medium (Fig. 9). After only a 1-min incubation of HeLa cells with 2-APB in a Ca^{2+}-free medium, the ionomycin-releasable Ca^{2+} pool was reduced by ~40%.

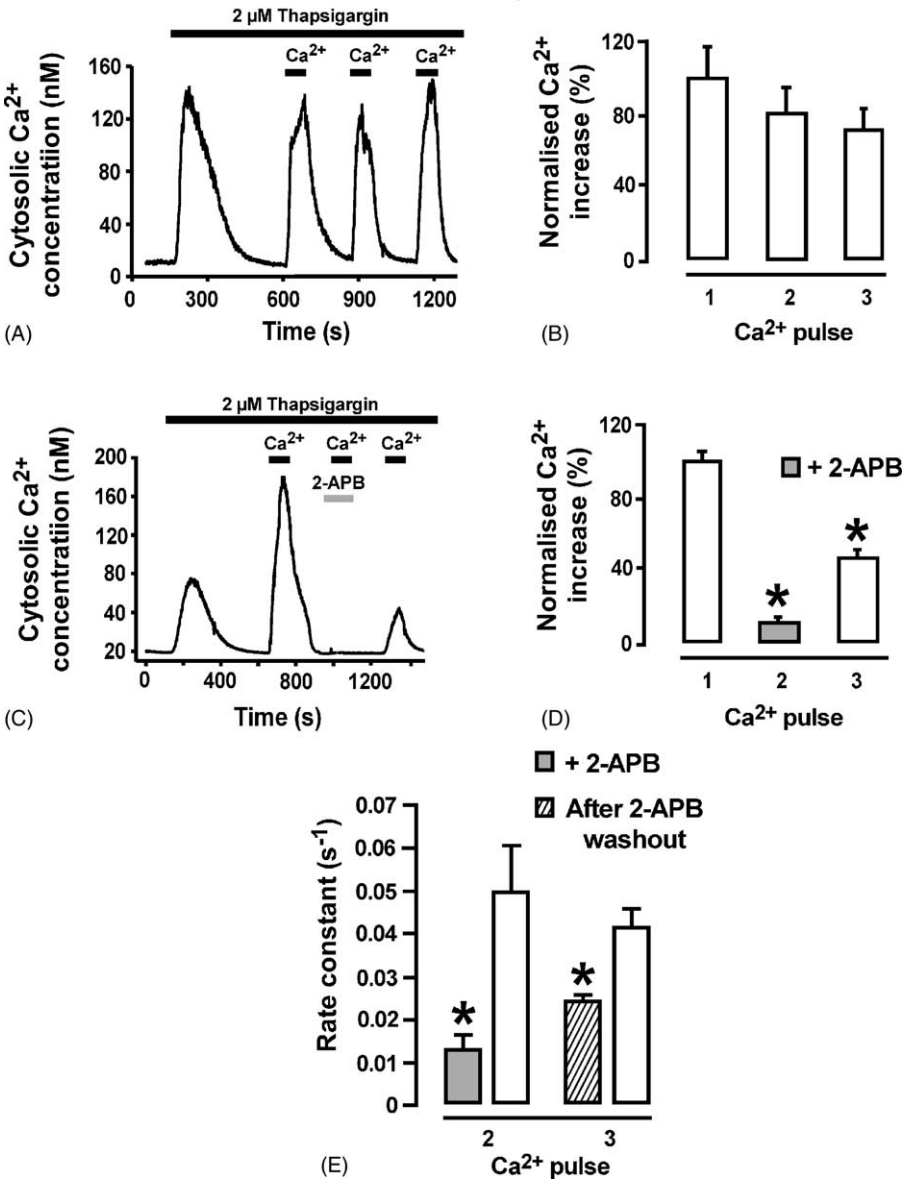

Fig. 5. 2-APB inhibits SOC-mediated Ca^{2+} influx. To activate SOC, the intracellular Ca^{2+} stores were discharged by incubation of cells with 2 μM thapsigargin in nominally Ca^{2+} free medium. Panel A shows the response of HeLa cells to repetitive activation of Ca^{2+} influx caused by the pulsatile re-addition of Ca^{2+} to the extracellular medium. Panel C depicts a similar experiment to A, except that 100 μM 2-APB was applied 1 min before and during the second Ca^{2+} pulse. Panels B and D summarise data from the experiments illustrated in panels A and C (mean ± S.E.M. for at least 30 cells from three independent experiments for each condition). Panel E depicts the averaged first-order rate constant for the recovery of the $Ca^{2+}{}_{cyt}$ signals following the second and third Ca^{2+} pulses for control cells (open columns), cells in the presence of 2-APB (grey column) and following 2-APB washout (shaded column). The data were obtained by fitting the decline of the Ca^{2+} signals after removal of extracellular Ca^{2+} to a single-exponential decay curve. The asterisk (∗) indicates significantly different from control ($P < 0.05$).

This increased loss of Ca^{2+}, above the passive rate of Ca^{2+} depletion, was maintained for up to 30 min (Fig. 9), by which time the ionomycin induced Ca^{2+} signals were small in amplitude.

3.3. 2-APB inhibits cardiac excitation–contraction coupling

Data from several studies have demonstrated that 2-APB does not affect voltage-activated Ca^{2+} channels or RyRs [10]—key components of cardiac excitation–contraction coupling (EC-coupling). However, we observed that 2-APB caused concentration-dependent effects on EC-coupling in ventricular cardiomyocytes. At concentrations ≤2 μM, 2-APB did not have any apparent effect on electrically-evoked Ca^{2+} transients (Fig. 10B). With 10 μM 2-APB, there was a biphasic effect: a transient increase in the amplitude of systolic Ca^{2+} rises was followed by a progressive diminution of the electrically-evoked responses (Fig. 10C). Concentrations of 2-APB that were ≥50 μM caused a simple decline

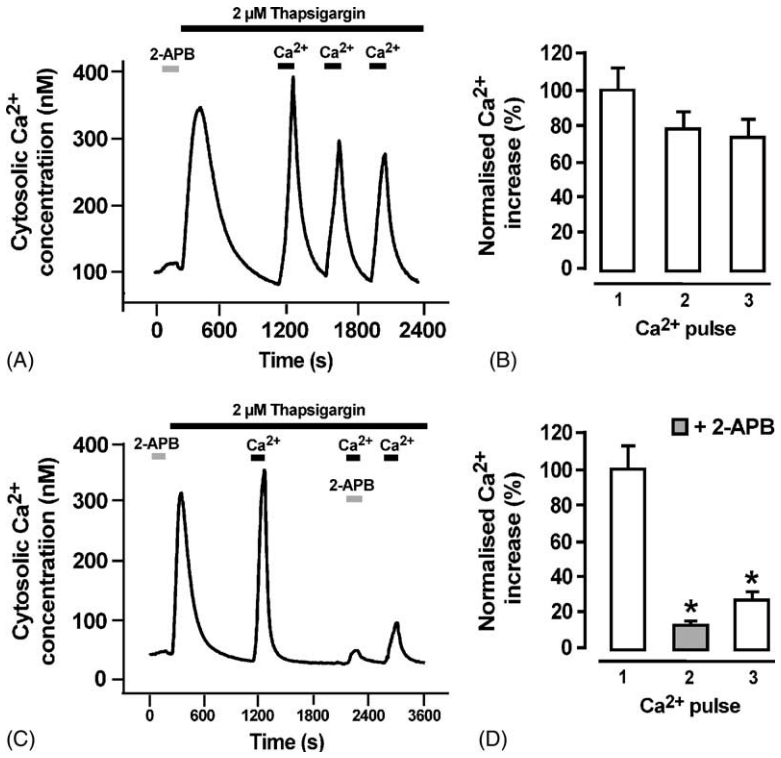

Fig. 6. The inhibition of SOC-mediated Ca^{2+} entry by 2-APB is use-dependent. Panel A shows the lack of effect of 2-APB on subsequent Ca^{2+} influx if it is applied before activation of SOC. Panel C depicts the potent inhibition of SOC-mediated Ca^{2+} influx if 2-APB is applied after activation of SOC. Panels B and D summarise data from the experiments illustrated in panels A and C, respectively (mean ± S.E.M. for at least 30 cells from three independent experiments for each condition). The asterisk (∗) indicates significantly different from control ($P < 0.05$).

in the amplitude of the electrically-evoked Ca^{2+} transients, until signals could no longer be generated (Fig. 10D and E).

4. Discussion

Since its introduction, 2-APB has been used in many studies to demonstrate the participation of $InsP_3$Rs in intracellular signalling. The relatively low cost, rapid membrane permeability in some cell types and apparent lack of effect on RyRs [10] makes it potentially more useful than other antagonists of $InsP_3$-induced Ca^{2+} release such as xestospongins or heparin. However, since the original study by Maruyama et al. [9], it has become clear that 2-APB can affect multiple processes involved in cellular Ca^{2+} homeostasis. Despite the lack of specificity, it remains worthwhile characterising the properties of 2-APB to investigate whether it has any utility for investigating aspects of Ca^{2+} signalling.

The complete inhibition of Ca^{2+} mobilisation evoked by the $InsP_3$ ester (Fig. 1) indicates that 2-APB can readily cross the plasma membrane of HeLa cells. 2-APB also gave a rapid inhibition of histamine- and ATP-evoked Ca^{2+} signals (Figs. 2 and 3), although there was an unexpected discrepancy in the ability of 2-APB to abrogate responses to these two agonists. 2-APB was more effective in inhibiting ATP-evoked Ca^{2+} signals than those generated by histamine. With all ATP concentrations tested (1–100 μM), 100 μM 2-APB fully inhibited its effect (Fig. 3). However, in the presence of 100 μM 2-APB, HeLa cells were able to respond to submaximal histamine concentrations, which gave lesser Ca^{2+} signals than a maximal ATP dose, albeit that the number of responsive cells and the amplitude of the Ca^{2+} signals were reduced (Figs. 2 and 3E). The reason why 2-APB was able to more effectively inhibit ATP-evoked Ca^{2+} signals may be due to an additional antagonist effect on the purinergic receptors. Alternatively, it may indicate that histamine stimulates the production of additional Ca^{2+}-releasing messengers in addition to $InsP_3$, which are 2-APB insensitive.

The rapid on-set of inhibition by 2-APB contrasts with its slow reversibility. Even after 5 min of continual perfusion, histamine- and ATP-evoked Ca^{2+} responses did not recover (Figs. 2 and 3). This long-lasting inhibition of Ca^{2+} signals was only apparent if 2-APB was applied during cell stimulation. When given on its own, 2-APB had little effect on subsequent responses (Fig. 4), in keeping with a 'use-dependent' effect. Several previous studies have noted a slow reversibility of 2-APB's effects in the order of tens of minutes [14,19–21], whilst others have demonstrated that it reverses within a few minutes [17] or even immediately [24].

We did not identify all the cellular sites in HeLa cells to which 2-APB will bind and slowly reverse. However, it is clear that it can bind to activated SOC channels (Figs. 5 and 6), and persist for several minutes (Fig. 5D). The pro-

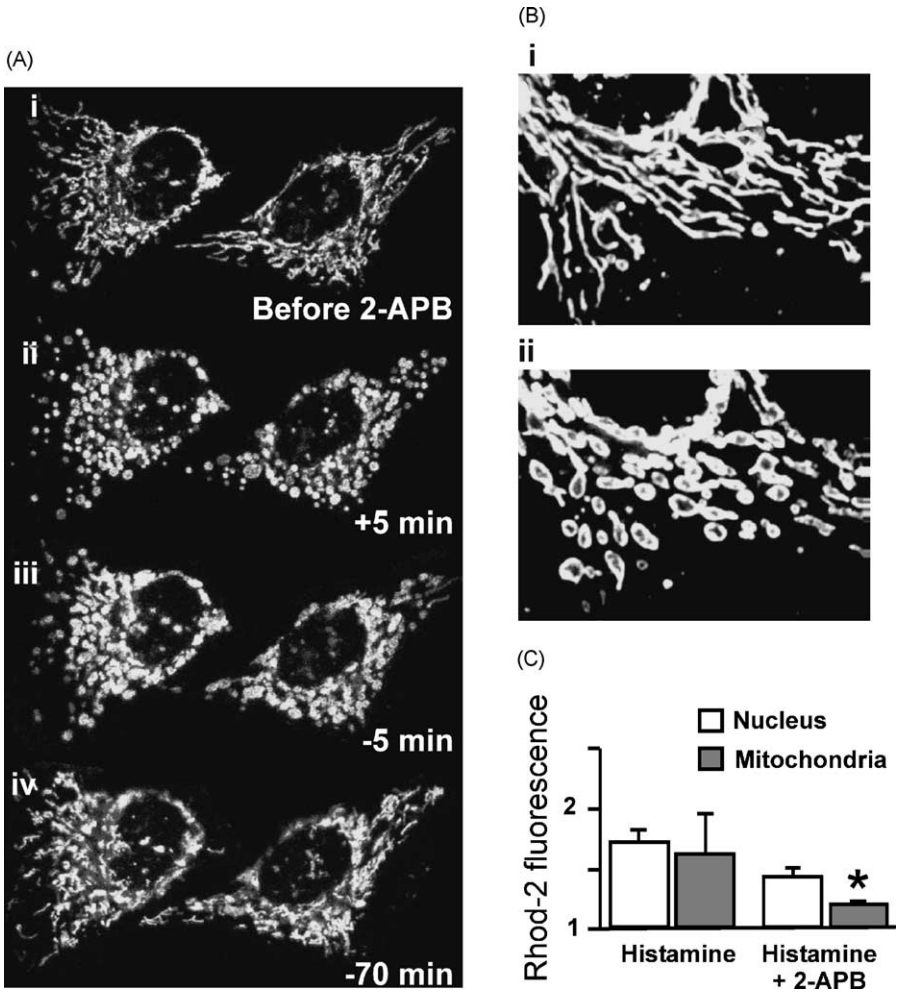

Fig. 7. 2-APB causes mitochondria to swell, but not change shape and inhibits Ca^{2+} uptake. The sequence of images in panel A show that mitochondria retain their membrane potential during perfusion with 100 μM 2-APB. HeLa cells were loaded with TMRE as indicated in Section 2, and imaged with a BioRad MRC1024 laser scanning confocal microscope (excitation 514 nm, emission 525LP). Images were acquired (i) before, (ii) 5 min after addition of 100 μM 2-APB, and then (iii) 5 min, and (iv) 70 min after washout of 2-APB with fresh extracellular medium. 2-APB was left on the cells for 15 min before washout. There is no loss of TMRE fluorescence at any stage, indicating that the mitochondria do not depolarise. Panel B shows a portion of a HeLa cell before (i) and after (ii) perfusion with 100 μM 2-APB for 5 min. Cells were stained with Mitotracker Green FM (0.1 μM, 20 min) and imaged with a BioRad MRC1024 laser scanning confocal microscope (excitation 488 nm, emission >500 nm). Panel C illustrates that mitochondrial Ca^{2+} sequestration is reduced by 100 μM 2-APB. Cells were loaded with Rhod-2 (1 μM; 30 min). Rhod-2 fluorescence was monitored in the nucleus and mitochondria using a BioRad 1024 confocal microscope to provide a simultaneous measure of cytosolic and mitochondrial matrix Ca^{2+} concentration, respectively (nuclear and cytosolic Ca^{2+} mirror each other in HeLa cells). The bars show the fold increase in Rhod-2 fluorescence over basal signal (i.e. F/F_o) following application of 100 μM histamine. The data represent the averaged peak Rhod-2 signal ± S.E.M. ($n = 3$). The asterisk (∗) indicates significantly different from control ($P < 0.05$).

longed inhibition of SOC-mediated Ca^{2+} entry almost certainly contributed to the lack of recovery of histamine and ATP-evoked Ca^{2+} signals after washout of 2-APB, as the intracellular Ca^{2+} stores would not have fully recovered between agonist pulses (Fig. 8). It is plausible that 2-APB reverses slowly from $InsP_3Rs$ and SERCAs, and that this also prevented the recovery of agonist responses.

The observation that 2-APB slowed the recovery of $Ca^{2+}{}_{cyt}$ transients caused by SOC-mediated Ca^{2+} entry (Fig. 5E) indicated that it inhibited processes responsible for restoring basal Ca^{2+} levels. It is known that 2-APB can inhibit SERCA pumps [11,26]. However, in the experiments shown in Fig. 5, the SERCA pumps were inhibited by several minutes of perfusion with thapsigargin prior to 2-APB addition. The ability of 2-APB to reduce the rate of recovery of $Ca^{2+}{}_{cyt}$ signals in the presence of thapsigargin indicated that it affected other Ca^{2+} removal mechanisms, such as PMCAs and mitochondria. Clearly, 2-APB does interact with mitochondria, since it cause them to swell (Fig. 7A) and inhibits extrusion of Ca^{2+} [19]. Although it did not evoke mitochondrial depolarisation (Fig. 7B), their ability to sequester Ca^{2+} was reduced (Fig. 7C).

The effect on SERCA pumps probably caused the run-down of the Ca^{2+} stores observed when 2-APB was

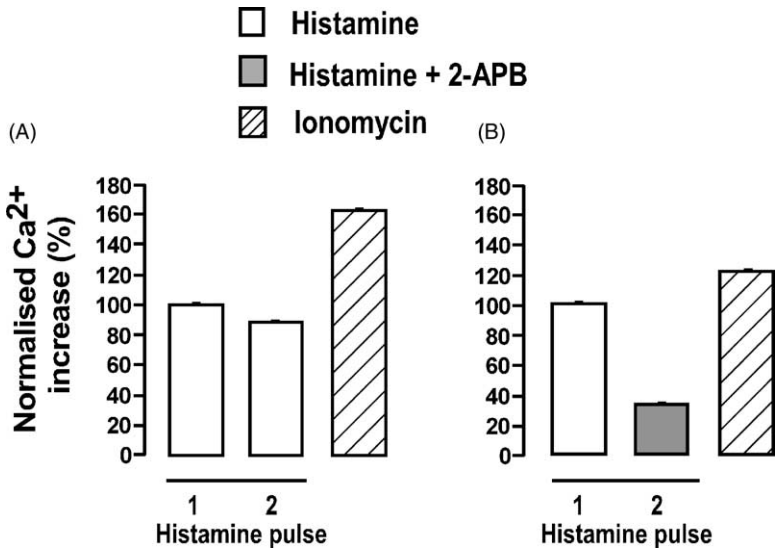

Fig. 8. 2-APB inhibits the refilling of the intracellular Ca^{2+} pool. Panel A depicts the averaged response of HeLa cells to two pulses of 100 μM histamine in Ca^{2+}-containing extracellular medium (1 min application and 10 min washout) followed by addition of 10 μM ionomycin in Ca^{2+}-free extracellular medium to asses the relative size of the intracellular Ca^{2+} pool. Panel B depicts a similar experiment to that shown in panel A, except that 100 μM 2-APB was added 1 min before and during the second histamine application. For both panels A and B, the data represent mean ± S.E.M. for at least 30 cells from three independent experiments.

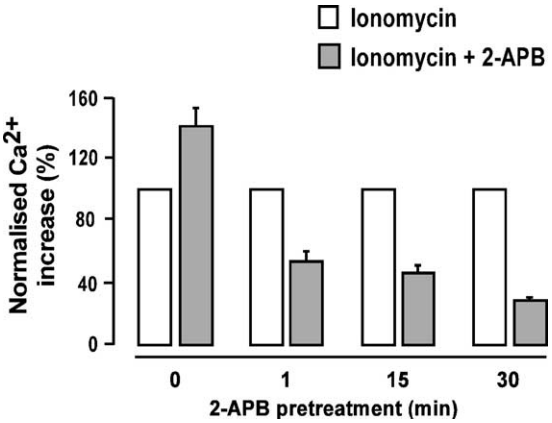

Fig. 9. 2-APB causes the gradual depletion of intracellular Ca^{2+} stores. The bars depict the normalised responses of HeLa cells to 10 μM ionomycin (applied in Ca^{2+} free medium) after incubation in Ca^{2+}-free medium without (open columns) or with (grey columns) 100 μM 2-APB. The duration of pre-treatment with 2-APB prior to ionomycin addition is shown beneath the columns. The data represent mean ± S.E.M. for the ionomycin-evoked peak Ca^{2+} transient amplitude, with at least 30 cells analysed for each time point.

applied on its own (Fig. 9). The ability of 100 μM 2-APB to deplete ~40% of the ionomycin-releasable Ca^{2+} pool after only 1 min of incubation suggests that it dramatically inhibits Ca^{2+} sequestration. This depletion of the intracellular Ca^{2+} pool occurred in the absence of a detectable Ca^{2+}_{cyt} rise. Clearly, 2-APB cannot be used as a diagnostic for the involvement of $InsP_3Rs$ in the generation of cellular Ca^{2+} signals if it simply causes the passive release of intracellular Ca^{2+} stores (Fig. 9).

An effect of 2-APB on SERCA pumps can explain the progressive inhibition of EC-coupling in ventricular cardiomyocytes (Fig. 10). The biphasic effect of 10 μM 2-APB on the amplitude of the evoked Ca^{2+} signals most probably reflected an initially enhanced response due to incomplete Ca^{2+} sequestration following a depolarisation-evoked Ca^{2+} signal, with the lack of Ca^{2+} store refilling eventually leading to a failure of EC-coupling. With the higher concentrations of 2-APB, a more pronounced inhibition of SERCAs would have caused the immediate run-down of the Ca^{2+} stores and corresponding Ca^{2+}_{cyt} responses. Although cardiac myocytes can produce $InsP_3$ in response to some inotropic stimuli [28], it is not thought to play any role in normal EC-coupling. Furthermore, SOC is also not believed to play a significant role in ventricular cardiomyocyte EC-coupling.

Given that 2-APB has so many intracellular targets, it is important to consider if it has any significant use at all. In a previous study, we used 2-APB to investigate whether $InsP_3$ mediated the inotropic or pro-arrhythmogenic effects of endothelin on atrial myocytes. By empirically investigating the effect of a range of 2-APB concentrations similar to that shown in Fig. 10, we were able to find a dose (2 μM) that did not affect EC-coupling, but could inhibit responses to the $InsP_3$ ester [28]. Therefore, in some cell types adjusting the concentration of 2-APB may provide a window of opportunity where the effect of 2-APB will be relatively more specific for $InsP_3Rs$ over other processes involved in Ca^{2+} handling. Furthermore, 2-APB may be useful in distinguishing between different pathways of Ca^{2+} release and entry. We have observed that 2-APB does not prevent Ca^{2+} release or Ca^{2+} influx stimulated by arachi-

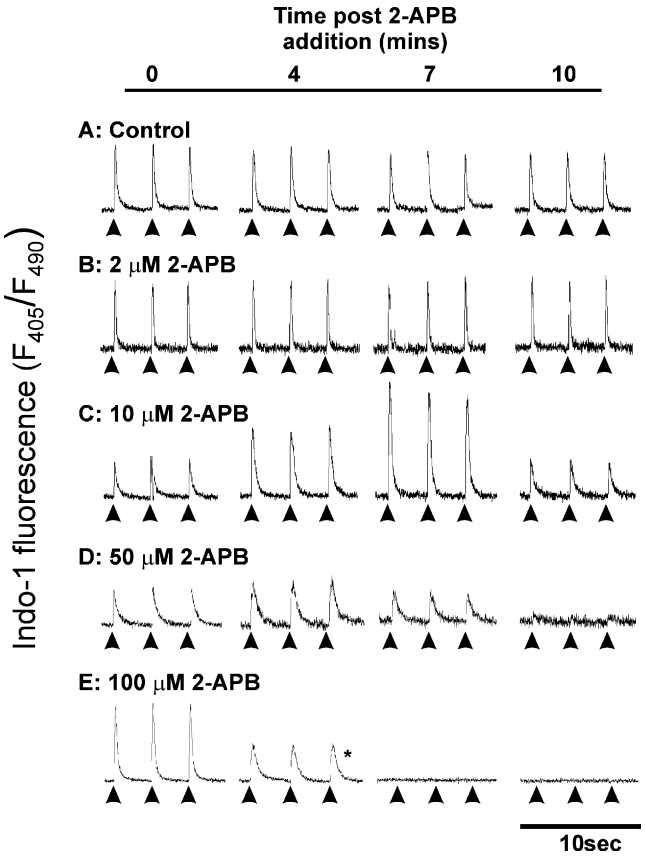

Fig. 10. 2-APB modulates Ca^{2+} transients during excitation contraction-coupling. Isolated ventricular cardiomyocytes were incubated with the concentrations of 2-APB shown on the individual panels (A–E) and continuously electrically stimulated at 0.3 Hz (marked by arrowheads). The electrically-evoked Ca^{2+} transients were recorded using ratiometric Indo1 measurements (see Section 2). For each 2-APB concentration, the effect of 2-APB was assessed by monitoring 10-s recordings of Indo1 fluorescence at the times indicated at the top of the figure. Between recordings, the electrical pacing was maintained, but the UV illumination of Indo1 was removed to prevent photodamage or bleaching of the indicator.

donate (data not shown), consistent with observations using HEK-293 cells [32]. Several other non-voltage-activated Ca^{2+} entry pathways that are distinct from SOC are also not inhibited by 2-APB, including maitotoxin-evoked Ca^{2+} entry in hepatocytes [17], *S*-nitrosylation-induced influx in a smooth muscle cell line [33], Ca^{2+} influx caused by diacylglycerol analogues in PC12 cells [34], muscarinic activation of non-selective cation channels in smooth muscle [35]. In many instances, 2-APB can be used to block SOC-mediated Ca^{2+} influx, although it should be noted that it is not completely specific for SOC [10]. The ability of 2-APB to penetrate into different cell types appears to vary significantly [13]. This could explain the high sensitivity of some cell types to 2-APB [36] and the lack of effect 2-APB on $InsP_3$-evoked Ca^{2+} release in others [37].

Clearly, 2-APB may be useful in probing different pathways of Ca^{2+} release and entry. For some cells, it is a rapidly membrane-permeant blocker of $InsP_3Rs$, and completely inhibits SOC-mediated Ca^{2+} influx. However, it is necessary to rigorously investigate the effect that 2-APB has on Ca^{2+} homeostasis in the cells under examination. The prevention of Ca^{2+} signals by 2-APB may not necessarily indicate a role for $InsP_3Rs$, since 2-APB can cause an imperceptible depletion of intracellular Ca^{2+} stores. In addition, it can affect other cellular processes, such as mitochondrial morphology, which may indirectly alter cellular Ca^{2+} signalling. Furthermore, the effects of 2-APB may be difficult to reverse.

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